HAYATI Journal of Biosciences, December 2008, p 135-139 Vol. 15, No. 4
ISSN: 1978-3019

Virgin Coconut Oil (VCO) Enriched with Zn as Immunostimulator for Vaginal *Candidiasis* Patient

HERY WINARSI[1*], HERNAYANTI[1], AGUS PURWANTO[2]

[1]Biology Faculty, Jenderal Soedirman University, Karangwangkal Campus, Purwokerto 53122, Indonesia
[2]Margono Soekarjo Hospital, Jalan dr. Gumberg Street No. 1, Purwokerto 53146, Indonesia

Received February 4, 2008/Accepted November 4, 2008

Disturbance on the immune system and deficiency of Zn is two factors which often trigger vaginal *candidiasis* patient. The aim of the research was to study the effect of virgin coconut oil (VCO) enriched with Zn to the amount of neutrophil and lymphocyte subset cells, and the level of IL-2 and IgG in vaginal *candidiasis* patient. Thirty women were grouped into three (ten women in each group): A, B and C, and intervened for two months. Women in A group were intervened with two tablespoon/day; those in B group were intervened with one tablespoon/day; while those in C group served as control (placebo). Blood was sampled at baseline time, one and two months after intervention. Hematological test by Micros-OT was done on a part of blood, and the plasma was used for IL-2 and IgG level tests using ELISA. The virgin coconut oil enriched with Zn maintained the number of neutrophil and NK cells, but increased Tc cells from 521 to 649 cells/mm^3, increased Th cells from 1.090 to 1.380 cells/mm^3. The enriched VCO also increase level of IL-2 from 0.25313 to 0.27337 pg/ml, while the IgG level changed from equivocal to negative. The recommended dosage was one tablespoon each day.

Key words: IL-2, IgG, neutrophil, NK, Th and Tc-cells, VCO, Zn

INTRODUCTION

Vaginal *candidiasis* is a pathologic condition marked by excessive production of mucus from vaginal vulva. Disturbance on the immune system is often trigger the *candidiasis* women (Jawetz *et al*. 1996). Several factors causing disturbance on the immune system include low level of antioxidant status (Winarsi *et al*. 2008a), high level of body-free-radicals, bad nutritional status (Winarsi *et al*. 2005a), and Zinc (Zn) deficiency (Winarsi *et al*. 2005b). Some clear symptoms of Zn deficiency are increasing to infection, disturbance on the function of T-lymphocyte to produce IL-2, and on the function of B-lymphocyte to produce antibody (Mocchegiani & Muzzioli 2000). Zn is important for the activity of superoxide dismutase (SOD) enzyme which is significant for defence against free radicals (Davis *et al*. 2000; Winarsi *et al*. 2005b). Rook in Roitt (1996) stated that aside from T cell, the immunity of *candidiasis* vagina patient caused by *Candida albicans* is also affected by neutrophil.

Commont drugs for *candidiasis* can cure it, but often shows immunocompromise effect. Therefore, the use of natural substance, such as *Virgin Coconut Oil* (VCO) is a good choice. Several researchers reported the potential of VCO as antifungal, antibacterial and antiviral agent (Bergsson *et al*. 1998), and for maintaining the immune system (Enig 2005). *In vitro* test showed that several fatty acids in VCO are potential as antibactery (Bergsson *et al*. 2001), antivirus (Bartolotta *et al*. 2001), and immunostimulant (Witcher *et al*. 1996), which are important to fight infection. This research aimed to explore the effect of VCO enriched with Zn to the amount of neutrophil, natural killer (NK), T-cytotoxic (Tc), T-helper (Th) cells, the levels of Interleukin-2 (IL-2), and Immunoglobuline-G (IgG) in vaginal *candidiasis* patient infected by *C.albicans*.

*Corresponding author. Phone: +62-281-638794, Fax: +62-281-631700, E-mail: winarsi@yahoo.com

MATERIALS AND METHODS

Thirty vaginal *candidiasis* patients were selected based on the following criterias: i.e. the number of *C.albicans* in the vaginal fluid (>10^5 cfu/ml), living in Purwokerto, voluntarily joint the research, and willing to sign an *informed consent* form. They were grouped into 3 groups, 10 patients in each group. Those in A group took orally 2 tablespoons of enriched VCO or 20 ml/day; those in B group took 1 tablespoon or 10 ml/day; while those in C group served as control (by placebo). The VCO was given for two months, and during the research conducted by recall food consumed as much as 8 times. Two ml of blood was sampled intravenously at baseline time, 1 and 2 months after intervention using venoject tube contains EDTA. Some of the whole blood was used for hematological test using Mikros-OT, while the plasma part was used for IgG (MBU diagnostic kits, 2006) and IL-2 test (Endogen Human IL-2 Elisa Kit, Pierce Biotechnology, Inc.). The number of neutrophil cell was estimated as 70% of granulocyte; NK cell = 10% of lymphocyte; Tc-cell =30% of T-lymphocyte; and Th cell = 60% of T-lymphocyte (Baratawidjaja 2000).

IgG Anti *C.albicans* Level Assay Using MBU Diagnostic Kits. Level of IgG anti *C.albicans* were determined by ELISA kits according to the manufacturer's instructions. Briefly, 100 µl of positive control was poured into wells A1 and B1, and 100 µl of negative control was poured into wells C1 and D1 each. One hundred µl of serum dilution buffer was added to well E1 for the control of conjugate. Ninety µl of serum dilution buffer and 10 µl of the assayed sera preliminary diluted

1:20 was added to the rest of the wells of the plate. Serum dilution buffer solution should be changed in color. The plate was closed and incubated for 30 min at 37 °C, before the wells were washed. One hundred µl of conjugate working solution was added to each well. The plate was added, and incubated for 30 minutes at 37 °C. One hundred µl of Tetramethylbenzidine (TMB) chromogen working solution was added to each well. The plate lid was closed for 20 minutes in a place away from light. Reaction was stopped by adding 50 µl of stop solution to each well. The absorbance of the solutions in each well was read at 450 nm. Level of IgG to *C.albicans* was analyzed qualitatively using Candida-IgG kit Medical Biological Union (2006), then read with ELISA. Absorbance of each sample was compared with a *cut off* value. The *cut off* (CO) value obtained was 0.766. The test was positive if sample absorbance value = CO + 15%, vice versa the test was negative if sample absorbance value was = CO – 15%, while sample absorbance value = CO $\pm$ 15%, was *equivocal* (intermediate).

IL-2 Level Assay using Endogen Human IL-2 Elisa Kits. Level of IL-2 were determined using ELISA kits according to the manufacturer's instructions. Briefly, 50 µl of reconstituted standards or samples in duplicate was added to each well. Fifty µl of biotinylated antibody reagent was added to each well. Fifty µl of standard diluent was added to all wells containing no standards or samples. They were carefully covered and incubated for 3 hours at room temperature, before being washed. One hundred µl of prepared streptavidin-HRP solution was added to each well. They were covered and incubated for 30 minutes at room temperature, before being washed. One hundred µl of TMB substrate solution was pipetted into each well. Enzymatic color reaction was carried out at room temperature in the dark for 30 minutes. The substrate reaction yields, a blue solution that turns yellow when the stop solution is added. After 30 minutes, the reaction was stopped by adding 100 µl of stop solution to each well. Evaluation of the plate was performed within 30 minutes after stopping the reaction. Absorbance was measured at 450 nm, if 550 nm is not available.

Hematological Test by Mikros-OT. One ml blood was pushed by high pressured air through capillary into chamber. These reagents, i.e minidil and minilyse in certain amount were absorbed into chamber and mixed automatically. Minilyse is a reagent used for leucocyte lysis (leucocyte lysing reagent) in the cells calculation. Minidil is an isotonic buffer solution used for measurement of blood cells and hematocrit. Blood sample and reagent were absorbed on chamber, through micro-aperature which have been emited by a stream electrics current. The bood cells cause the electrics vibration have different amplitude and translated as element in all kinds of blood cells. There are many of sum and vibration yielded with size and number of cells. Miniclean is an enzymatic solution with proteolitic activity to clean blood cells calculator after being used. Blood volume in venoject-tube minimal injected to Mikros-OT device is 50 ìl, while analyzed only 10 ìl read by Micros-OT during 20 seconds.

RESULTS

The Number of Neutrophil, NK, Tc, and Th cells. At the baseline time, the number of neutrophil showed no differences between groups (P = 0.73) which is 3.822-5.649 cell/mm^3. It means, all the samples are in the normal range (Table 1). Similar case was observed after one and two months following the treatment, all groups did not reveal differences in the number of neutrophil. The control group had total neutrophil above the normal range, while the treatment group is the normal range. Among the treatment groups, there were no differences (P = 0.71) although, the treatment was prolonged until the end of the second month.

The number of NK cells at the baseline time was not different among different groups (P = 0.75). This case was also observed after one month (P = 0.26) and two months after the treatment (P = 0.63) (Table 1). There was no differences in the number of Th-cell at the baseline time among the three groups (P = 0.75). No differences was observed at one month following intervention. Two months after the treatment, however, the effect was significantly detected (P = 0.04), where the treatment group receiving 1 tablespoon enriched VCO/day, showed an increase in Th-cell from 1.090 to 1.380 cells/mm^3.

Tc cell is a subset of T cell, and the amount is 30% of the T-cell. At the beginning of the research, the number of Tc cell was not different among the groups (P = 0.75). One month treatment did not affect the different groups. Two months after treatment, however, group given with 1 tablespoon/day was significantly different (P = 0.04) from the control group, the number of Tc cell increased from 521 to 649 cells/mm^3.

Level of IL-2. In this research, IL-2 level analyzed by using Elisa Human Endogen IL-2 kits at baseline time ranged from 0.248444 to 0.248458 pg/ml and was significantly lower than those of the normal rate (3.5 pg/ml). After one month, both the treatment groups (2 tablespoon/day and 1 tablespoon/day) were not different from the control group. However, after two months, there was a significant difference between the treatment groups and the control group. Between the two treatment groups, however, there was no difference (P = 0.99).

Table 1. The number of neutrophil, NK, Th and Tc cells of vaginal *candidiasis* patients intervened VCO enriched with Zn

Type of cells	Number of cell (cell/mm^3)									Number of normal cells (cell/mm^3)
	0			1			2			
	A	B	C	A	B	C	A	B	C	
Neutrophil	4110	3820	5650	3730	6970	3930	3930	4190	7280	840-4760
NK	223	240	242	221	234	260	221	239	224	120- 320
Th	1040	1120	1130	1030	1100	1210	1040	1380	1090	560-1494
Tc	520	560	565	516	547	606	518	649	521	280- 747

A: VCO enriched with Zn group, 2 tablespoon/d; B: VCO enriched with Zn group, 1 tablespoon/d; C: control group; 0, 1, and 2: period of intervention (month).

The level of IL-2 group receiving 1 tablespoon increased from 0.25313 to 0.273374 pg/ml (Figure 1).

Level of IgG to *C. Albicans*. At the baseline time, absorbance values of treatment groups were *equivocal* (0.773-0.863) and not different from the control group (P = 0.96). One month after the treatment the two treatment groups were still not different from the control group. However, after two months the group receiving 1 tablespoon of VCO showed a negative level (0.429) absorbance, significantly different (P = 0.046) from the control group with *equivocal* absorbance level (0.883) (Figure 2).

DISCUSSION

The number of neutrophil cell was normal, however the activity as immune cell may not be optimal, so both groups may easily got infection. *C. albicans* is one of the pathogenic fungus causing morbidity and mortality of an immunocompromise individual (Wenzel & Pfaller 1991). The main cell for immunity against non-specific fungi is neutrophil. It can release fungicidal material such as reactive oxygen and lysosome enzyme capable of killing fungi (Bimantara 1997). Romani (1999) stated that predisposition factors are neutropheny and low number of T_{CD4} - cell or Th-cell. This finding proves that VCO enriched with Zn suplementation maintains the number of neutrophil cells in normal range, so that its activity is optimal to fight *C. albicans*.

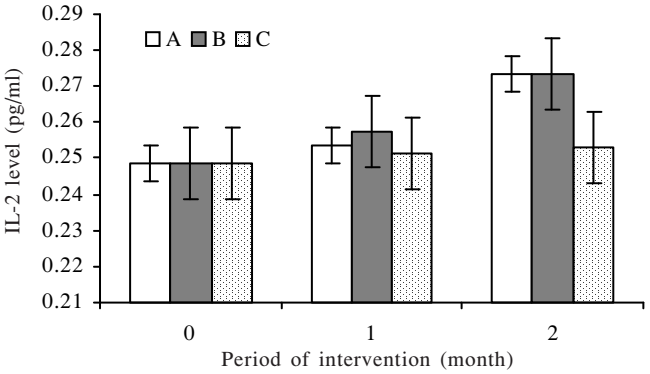

Figure 1. Effect of VCO enriched with Zn on IL-2 level. A: VCO enriched with Zn group, 2 tablespoon/d; B: VCO enriched with Zn group, 1 tablespoon/d; C: control group; 0, 1, and 2: period of intervention (month).

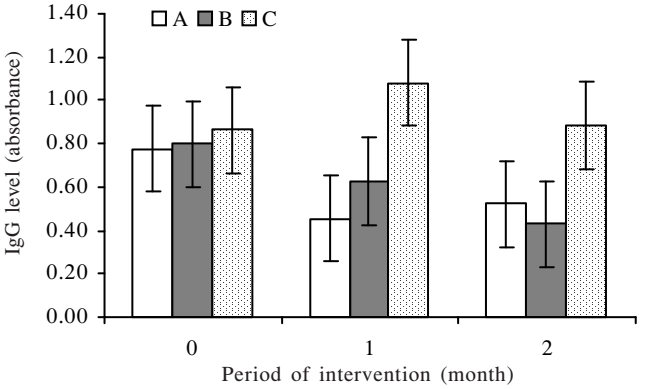

Figure 2. Effect of VCO enriched with Zn on IgG level. A: VCO enriched with Zn group, 2 tablespoon/d; B: VCO enriched with Zn group, 1 tablespoon/d; C: control group; 0, 1, and 2: period of intervention (month).

This supplement is capable to increase the number of Th and Tc cells, although still within the normal range. The function of Th cell is to help the cell recognizes infected cell, then activates other subset of T cells. Th cell also releases cytokine capable of activating macrophage. Tc cell will eliminate virus-infected-cell and destroy cancer cell (Romani *et al*. 1996). Therefore increasing Tc cell indicates that VCO enriched with Zn is potential as anticancer.

C. albicans is microorganism capable to live together with opportunist pathogen in human body. Releasing cytokine from immunocompetent cell is important for defence mechanism of host cell against fungi (Romani *et al*. 1996; Mencacci *et al*. 2000). Stimulation of human peripherical blood mononuclear cell (PBMC) by *Candida* or its antigen induces production of various cytokine. Healthy woman mononuclear cells produces TNF and IL-1, however stimulated mononuclear cell induces early and long term production of mRNA for IL-1β, TNF-á, and IL-6, as well as mRNA for IL-2, GM-CSF, and IFN-∂.

Several researchers reported that components of *C. albicans* cell wall has an immunomodulator effect on host defence system, i.e. cytokine. However the mechanism and the substance responsible for the effect is debated. Fungi and their antigens may induce the release of IL-2, IFN-ã, TNF-á, G-CSF, and in GM-CSF (Cenci *et al*. 1995). These cytokines may, in turn, activate or enhance the antifungal function of phagocytes against *Candida* (Roilides *et al*. 1995). Interleukine-1 is a type of cytokine enhancing Th_1- cell produces IL-2 (Witkin *et al*. 2000). This low level of IL-2 at the baseline indicated low status of subject immune system, so that did not optimal trigger replication of Th1 cell.

Wakui *et al*. (1986) reported that mushrooms ß-glucan had antitumor activity. The substance also showed immunomodulator effect on experimental animal immune system (Lee *et al*. 2001). *Candida* ß-glucan which is the *Candida* cell wall polysacharide, is also a potential immunomodulator in human body defence system. Nakagawa *et al*. (2003) argued that *Candida* ß-glucan suppreses production of type-1 cytokine, i.e. IL-2 and IFN-∂ on human PBMC culture. This effect will not appear if the number of PBMC monocyte decreases, in other words, ß-glucan is capable to suppress T cell cytokine. It indicates that ß-glucan suppresses monocyte as *innate* response through T cell.

Vaginal *candidiasis* is immunocompromise condition indicated by the activity of monocyte immunocompetent cells as well as T cells, giving rise to virulent character of *C. albicans* (Farah *et al*. 2001; Farah *et al*. 2002). Disturbance on Th_1 cell causes low host response to *C. albicans*, as well as low *candidiasis* recovery. This finding proved that a weak effect of ß-glucan on host inflamation is released as a response to *Candida* infection, which consequently brings about development of *candidiasis*. On the other hand, Ashman and Papadimitriou (1995) proposed that ß-glucan induces lymphocyte proliferation, production of IL-2, and IFN-∂, as well as NK cell cytotoxic activity.

Routine consumption of this supplement may increase the IL-2 level since Zn is needed for development of lymphocyte cell as immunocompetent cell. The VCO itself will also provides fast supply of energy without side effect on the levels of cholesterol (Winarsi *et al*. 2007), and blood sugar or body weight (Winarsi *et al*. 2008b). Although reasonably far from the normal level, VCO enriched with Zn showed a positive effect on Th_1 cell activities. Production of IL-2 by Th_1 cell affects the function of Th cell in inducing proliferation and differentiation of B cell to form memory cells as well as plasma cells to produce immunoglobuline.

Level of IgG to *C. albicans* baseline was indicated by the women condition which was easily attacked by *candidiasis,* especially when the immune system decreased and was not quickly improved. This research revealed clearly that the VCO enriched with Zn is capable to improve B cell activity to produce antibody. IgG is important for cellular immunity by damaging cellular antigen through interaction with complement system or cytolitic effect of Killer cell (K cell), eosinophil and neutrophil. K cell is an effector of *Antibody Dependent Cellular Cytotoxicity* (ADCC). ADCC capable to destroy various multicellular microorganisms as well as single cell, such as *C. albicans*. Antibody increases resistance to *C. albicans* infection, however high level of IgG indicates cronical infection[13]. Host resistance to *C. albicans* infection is generally thought to be established by Th_1, rather than Th_2 response (Kaposzta *et al*. 1998). This research observed that morbidity was associated with high IgG antibody titers, that humoral response is not critical to host defense against *C. albicans* during primary systemic infection.

Witkin *et al*. (2000) proposed that Th_1 cell also produces IFN-∂ which inhibits formation of *germ tube*. Type 1 hypersensitivity reaction correlates with Th_2 cell reactivity which gives rise to IL-4 and increases antibody production by B cell as well as releasing of PGE_2 (Prostagalandin E). PGE_2 inhibits proliferation and production of IL-2 from Th_1 cell but does not affect to Th_2 cell. Therefore, it can be predicted that PGE_2 does not have protective effect to *Candida*.

In conclusion, the treatment to vaginal *candidiasis* patients showed that the VCO enriched with Zn was potential as immunostimulator. However, it is recommended for vaginal *candidiasis* patient to consume the VCO enriched with Zn with a dossage of 1 tablespoon each day to optimise the immune status.

ACKNOWLEDGEMENT

The author thanks to *Ditbinlitabmas Dirjen Dikti* for funding support throughout this research through Research Funding Project 'Hibah Bersaing' XIV/1 and XIV/2, budget of year 2006 and 2007.

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